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The engineering of actinidin-encoding gene and its expression in *Saccharomyces cerevisiae*

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Abstract

Actinidin-encoding gene has been engineered and cloned in an expression-secretion vector designed for yeast *Saccharomyces cerevisiae*. The engineered actinidin-encoding gene consisted of DNA sequences encoding all N-terminal extension, mature actinidin, and part of C-terminal extension (without amino acids QR-encoding motifs). The variant of actinidin-encoding gene was then expressed in *S. cerevisiae* and analysed using immunoblotting technique. Analysis of expression suggested that no product of translation of the gene was detected. It was assumed that the gene may be expressed at transcription and translation level, but the expressed protein may be not be correctly processed and thus has been subjected to proteolytic degradation.

Keywords: actinidin – *Saccharomyces cerevisiae* – protein secretion

Introduction

Actinidin is a cysteine protease found in the fruit of Chinese goooseberry (*Actinidia chinensis*) or Kiwi fruit, constituting a polypeptide chain of 220 residues, having an Mr of 23.6 kDa (Carne and Moore, 1978; Baker, 1980). It is grouped in the class of plant thiol proteinases which includes papain, ficin and stem bromelain (McDowall, 1970; Baker, 1980). It resembles papain in that it catalyses the hydrolysis of benzoyl-L-arginine ethyl ester, has a broad range of optimum pH from 5 - 7 as well as substrate specificity (Johnson *et al.*, 1987). The crystal structure and amino acid sequence of actinidin are also very similar to that of papain with their catalytic sites virtually identical although differences do exist in their characteristics (Carne and Moore, 1978; Salih *et al.*, 1987).

The amino acid sequences (Carne and Moore, 1978) and the cDNA as well as the genomic DNA sequences of actinidin have been reported (Praekelt, 1987; Praekelt *et al.*, 1988; Snowden and Gardner, 1990). The first 20 amino acids of actinidin show hydrophobic characters typical of a signal sequence, suggesting that the protein enters the secretory pathway. Neither the subcellular location nor the physiological function of actinidin is known although it has been suggested that it may be involved in the defence against invading predators by releasing enzyme upon damage of the tissue (Praekelt *et al.*, 1988).

Several actinidin-encoding gene constructions have been created and expressed in *S. cerevisiae* (Yuwono, 1991). It was observed that the expressed actinidin was translocated into the vacuole. DNA sequence analysis suggested that the precursor actinidin contained amino acid sequence motifs within its N-terminal and C-terminal extensions that served as vacuolar translocation signals. Another study (Yuwono *et al., submitted*) also revealed that the removal of QR amino acids motif within the N-terminal extension and C-terminal extension, but keeping part of the C-terminal extension intact, resulted in the expression and secretion of actinidin into the culture supernatant. The protein, however, was not perfectly processed as it had smaller size to that of native actinidin. Therefore, it was of interest to explore further whether engineering the actinidin sequence at specific sequences would lead to expression and secretion of actinidin into the culture supernatant of *S. cerevisiae*.

Cloning and expression of the actinidin-encoding gene in yeast is of value because it may provide an alternative way to produce a proteolytic enzyme for food and industrial purposes, without having to purify it from the plant as in the case of papain. Expression and secretion may be carried out by using an expression-secretion vector (pYSV9) for yeast *Saccharomyces cerevisiae*. The vector contains the yeast a-factor promoter and signal sequences, *CYC1* terminator, and *LEU2* marker gene. The in-frame fusion of a foreign gene with the a-factor promoter will lead to a correct expression and secretion of the foreign protein. The expressed protein will finally be transported through the yeast secretion pathway using the a-factor signal sequences.

Materials and Methods

Microorganisms

Bacteria *Escherichia coli* DH5α [F', f80d*lac*ZDM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17(r_K^- m_K^+), *sup*E44, *rel*A1, *deo*R, D(*lac*ZYA-*arg*F) U169], was used as the host for cloning and preparation of recombinant DNA.

Yeast *S. cerevisiae* DBY746 (*MAT*α *his*3-D1 *leu2-3,112 ura3-52 trp1-289*) was used as the host for the expression of actinidin-encoding gene.

Plasmid

Plasmid used for the expression of the actinidin-encoding gene was pYSV9 (kindly supplied by Dr. Alan Mileham through Dr. Peter A. Meacock, Department of Genetics, University of Leicester, UK). This plasmid is an expression-secretion vector designed for *S. cerevisiae* system. It contains a leader sequence of *MF*α*1* required for regulating the expression of the cloned gene and directing the product of expression into secretion pathway.

PCR amplification of actinidin-encoding DNA

A variant of actinidin-encoding gene was created by PCR amplification targeted to a specific region using pEMBL-KIWI which carried the full-length actinidin cDNA (Yuwono, 1991) as the DNA template. Primers used for amplification were as follows:

(1) TW1: 5'- CC GAA GCT TTC AAC GCC AAA AAC TTG- 3', (2) P8: 5'- TTT GTC GAC TTA TCC ATC GTC TAC TCC- 3'. The amplified DNA fragment consisted of N-terminal extension (including the QRTN motif), mature actinidin, and C-terminal extension (without QR motif).

DNA manipulation and transformation. PCR-amplified actinidin DNA variant was subsequently cloned into the *Hind*III- *Sal*I sites of the pYSV9 vector to create an in-frame fusion with the yeast a-factor leader sequence present in the vector. The recombinant plasmid was then used to transform *Escherichia coli* DH5α using the heat-shock method. All cloning procedures were essentially as described by Sambrook *et al.* (1989). The recombinant actinidin gene construction, designated as pYSV-R4, was then used to transform yeast using lithium acetate-induced transformation method (Rose *et al.*, 1990).

Cultivation of yeast and precipitation of supernatant protein (Yuwono, 1991). The culture of *S. cerevisiae* carrying recombinant DNA was firstly grown at 30°C overnight with shaking in a YEPD broth medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose). The overnight culture was then used as the inoculum for the preparation of yeast culture of 100 ml volume for 24 hours at 30°C with shaking. Supernatant was harvested by centrifugation at 10,000 *g* followed by precipitation of the protein by employing three different methods of precipitation: (1) using ammonium sulphate at 50% saturation, (2) using cold acetone (1 volume of supernatant : 5 volume acetone), and (3) using the mixture of BSA (2 mg/100 ml supernatant) + 10% trichloro acetic acid (TCA). The mixture of supernatant was incubated at –20°C overnight, followed by centrifugation at 10,000 *g* at 4°C for minutes. The pellet obtained was then resuspended in 300 ml of distilled water.

Isolation of intracellular protein

Yeast transformant carrying recombinant plasmid harboring actinidin-encoding gene was cultivated in broth YEPD for 2 days, followed by pelleting the cell by centrifugation. Cell pellet was then resuspended in 1 ml of NaOH 0.25M, 1% mercaptoethanol, and incubated on ice for 10 min. Afterwards, 0.16 ml of 50% TCA was added and the incubation was continued for another 10 min on ice. The mixture was then centrifuged in an eppendorf microfuge at 10,000 *g* for 10 min. Pellet obtained was subsequently resuspended in 300 ml of distilled water.

Analysis of expression

Following precipitation, samples of supernatant protein and intracellular protein samples were electrophoresed on SDS-PAGE (Hames and Rickwood, 1990). Subsequently, proteins on the gel was detected by using immunoblotting technique. The first antibody used was a polyclonal antibody raised in chicken against purified actinidin extracted from Kiwi fruit. Detection of the antigen was carried out by using anti-chicken IgG alkaline phosphatase conjugate as the second antibody followed by colour development using nitroblue tetrazolium and bromocresol indolyl phosphate. All immunoblotting protocols were essentially as described by Yuwono (1991).

Results and Discussion

Amplification and cloning of actinidin-encoding gene

A variant of actinidin-encoding gene was constructed by PCR amplification of specific region of the full-length actinidin cDNA. The region amplified consisted of the N-terminal extension-coding sequence starting from amino acid number 24 (from the methionine initiating codon), thus including the QRTN motif, through the mature actinidin-coding sequence and the C-terminal extension-coding sequence up to amino acid number 374, that is just before QR amino acid motif. The scheme of the amplified region is

presented in Figure 1.

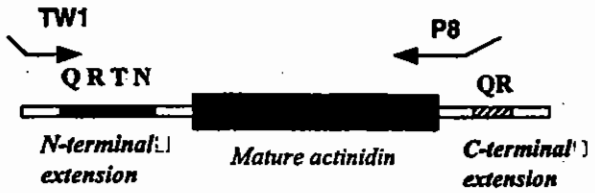

Figure 1. The scheme of amplified region of actinidin-coding DNA sequence. Primer TW1 was complementary to the DNA sequence starting from amino acid number 24, while primer P8 starting from amino acid number 374 which is located just before QR amino acid motif. QR(TN) is a putative vacuolar targeting signal.

The primers used for amplification of actinidin-coding DNA sequence was designed in a way that it included the restriction site for *Hin*dIII at the 5′ end of primer TW1, and the restriction site for *Sal*I at the 5′end of primer P8. This designed is intended to simplify the cloning procedure into pYSV9 vector (Fig.2).

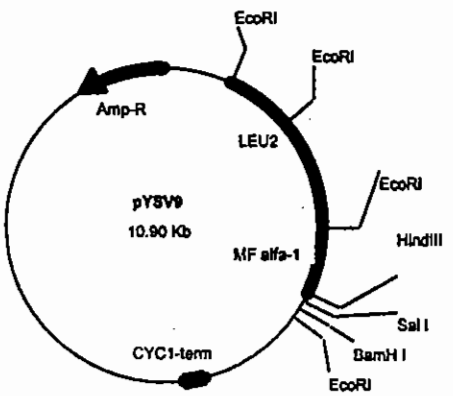

Figure 2. The scheme of plasmid pYSV9 used for expression and secretion of a foreign protein in *S. cerevisiae* as also employed in this study. The promoter region lies within the region designated as MF alfa-1 which includes a pre-pro leader sequence of *MFα1* mating type factor. Terminator used was derived from *CYC1* gene terminator (CYC1-term). The foreign gene, actinidin-encoding gene, was inserted between *Hin*dIII – *Sal*I sites.

The *Hin*dIII site is actually part of the proteolytic processing site of the *MFα1* leader peptide. The cloning of a foreign gene into this site will create the in-frame fusion with the *MFα1* leader peptide which result in a fusion polypeptide with α-factor destined to the secretion pathway. The fusion peptide will then be processed by a *KEX2*-encoded enzyme at the Glu-Ala sequence, which includes the *Hin*dIII site, and subsequently will be further processed by dipeptidyl aminopeptidase (DPAPase A) encoded by *STE13* gene. In this way, the foreign polypeptide will, in theory, be directed to the secretion pathway.

The result of PCR amplification resulted in a single band. The size of the amplified DNA fragment (designated as fragment R4) is 1050 bp plus several bases which constituted the restriction enzyme sites. The amplified fragment was then cloned into pYSV9 plasmid at the *Hin*dIII and *Sal*I sites and resulted in a recombinant palsmid designated as pYSV9-R4. Figure 3 shows the result of restriction analysis of the recombinant DNA by using *Eco*RI. It is clearly demonstrated that the recombinant DNA was digested into four fragments as there are four *Eco*RI sites within pSVY9. The actinidin-coding DNA sequence (fragment R4) fused with the smallest *Eco*RI fragment of the pYSV9 and thus created a bigger fragment which, as a consequence, had a close size to the third biggest fragment (of ca. 2000 bp) of pYSV9.

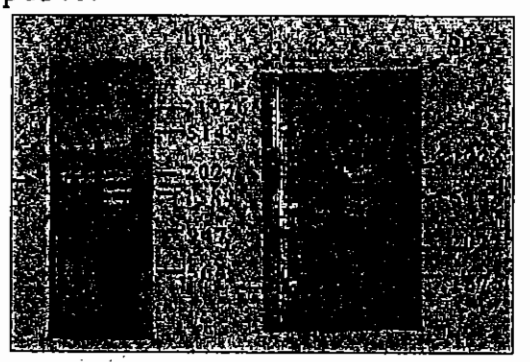

Figure 3. Restriction analyis of recombinant plasmid (pYSV9-R4) harboring a variant of actinidin-coding DNA sequences. 1 and 4: pYSV9-R4 digested with *Eco*RI, 2 and 5: 1 DNA digested with *Eco*RI and *Hin*dIII, 3: pYSV9 digested with *Eco*RI. Arrow on lane 1 shows double bands of DNA of close sizes in which the actinidin-coding DNA sequence was ligated to the smallest *Eco*RI fragment of pYSV9 (see arrow on lane 3).

The vector DNA (pYSV9) which had no foreign DNA insert was also digested into four fragments in which the smallest fragment was the fragment that fused with the R4 fragment. The recombinant DNA was subsequently used for transformation of yeast *S. cerevisiae.*

Analysis of expression

Transformant of *S. cerevisiae* harboring recombinant engineered actinidin-encoding gene was cultivated in a complete medium (YEPD) to drive the expression of the gene. Supernatanat was collected and precipitated by using different methods, i.e.: (1) ammonium sulphate, (2) cold acetone, and (3) BSA + TCA. The pellets were then resuspended in distilled water. Samples of concentrated supernatant were subsequently electrophoresed on an SDS-PAGE (Fig.4).

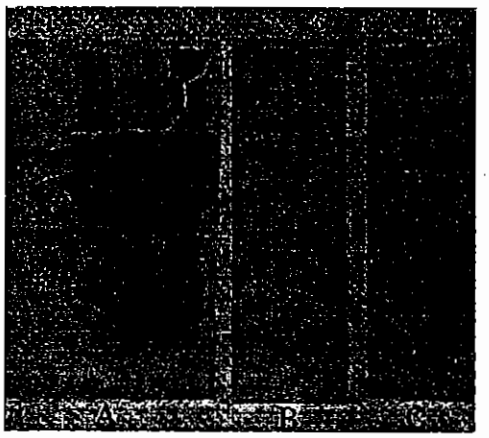

Figure 4. SDS-PAGE of supernatant of yeast culture precipitated using different methods: (A) BSA + 10% TCA, (B) ammonium sulphate 50%, (C) cold acetone 5X volume. 1: purified actinidin, (2, 4, 6, and 8): supernatant of yeast harboring recombinant plasmid pYSV9-R4, (3, 5, and 7): supernatant of yeast harboring plasmid vector pYSV9 without any inserted gene. Arrow shows a putative protein band corresponding to the actinidin-encoding gene present in recombinant plasmid pYSV9-R4.

It is shown on Figure 5 that different method of precipitation gave different results. Precipitation of supernatant proteins by using 50% ammonium sulphate and cold acetone did not result in clearly identifiable bands of protein. On the other hand, precipitation using BSA and TCA resulted in the emergence of sharp protein bands. On lane 4 (see arrow) a band of slightly smaller in size to that of purified actinidin was clearly detected. This protein band was absent from supernatant proteins precipitated from the culture harboring the vector plasmid which had no actinidin-coding DNA sequence inserted (lane 3).

To confirm the observation, either extracellular or intracellular proteins preparation were electrophoresed on an SDS-PAGE. The results (Fig.6) demonstrated that the supernatant of yeast culture harboring actinidin gene construction (pYSV9-R4) showed a band of protein having slightly smaller size to that of purified actinidin (see arrow on lane 2). The band, however, was not clearly identifiable as it was masked by the bulk of BSA proteins. In the intracellular protein preparation, no distinct band of protein corresponding to the native actinidin was detected.

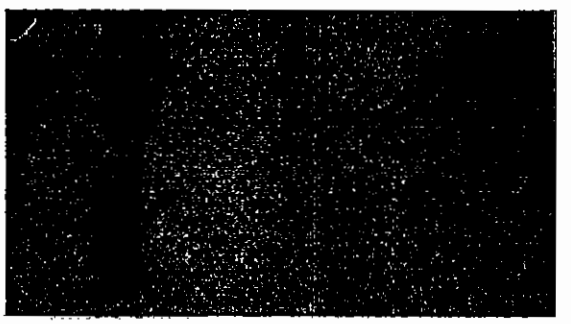

Figure 5. SDS-PAGE of intracellular and extracellular proteins of yeast.

1: Extracellular proteins of yeast carrying vector plasmid pYSV9
2: Extracellular proteins of yeast carrying recombinant plasmid pYSV9-R4
3: Marker proteins
4: Purified actinidin
5: Intracellular proteins of yeast carrying vector plasmid pYSV9
6: Intracellular proteins of yeast carrying recombinant plasmid pYSV9-R4
7: Intracellular proteins of yeast without any plasmid.

The results of immunoblotting, however, did not show any signal of positive immunoblotting reaction between anti-actinidin antibody with those protein bands detected on SDS-PAGE. The lack of positive immunoblotting reaction may be due to alteration of the protein structure as the precursor actinidin, if any, might not be correctly processed. Therefore, further work is now underway to confirm the presence of recombinant engineered actinidin secreted into the culture supernatant of yeast carrying actinidin-encoding gene construction.

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